DESCRIPTION

"METHOD AND APPARATUS FOR ANALYIZING BIOLOGICAL TISSUES"

The present invention relates to a method and an

5 apparatus for processing images of irregularly shaped
objects, such as biological tissues and items, in
particular of human or animal origin. The metric
quantification of a biological body part or tissue or of
a material spot or aggregate of any origin which is

10 contained therein is also performed by means of the
invention method. In particular, the method of the
present invention is applied to the "confocal
microscopy" technique.

The Laser Scanning Confocal Microscopy (LSCM) is a

15 known technique used for obtaining high resolution
images and 3D-images of biological specimens. LSCM is
based on a laser light beam which is focused on a point
or a small spot of a fluorescent specimen by means of an
objective lens. The laser beam is made to scan the

20 specimen through a x-y deflection mechanism. Both the
reflected and the emitted fluorescent light are focused
onto a photomultiplier via a dicroic mirror. The dicroic
mirror lets the fluorescent light to pass toward the
photomultiplier, through a confocal aperture (pinhole).

25 The out-of-focus light, coming from points that are not

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within the focal plane of the observed specimen, is stopped by the pinhole, while the focal plane information is recorded as a digital image. The intensity of the fluorescent light corresponds to a

5   pixel intensity (normally, as a 8-bit grey scale). By moving the microscope stage up and down, scanning in the z direction is effected, which allows for a 3D-reconstruction of the observed item. The digital image is then processed by suitable image digital filters

10 (contrast and brightness adjustement, noise removing, colour adding, etc.) and finally analysed.

Further improvements of the LSCM technique have brought to the Scanning Laser Ophtalmoscopy (SLO), which provides for a retinal imaging by direct observation of

15 the patient's eye through a scanning laser confocal microscope wherein the optics of the eye have the same function of the objective lens.

Confocal scanning microscopes which make use of normal visible light instead of laser light are also

20 known and are commonly used for corneal imaging.

The confocal ophtalmoscopy is a powerful tool for studying the living human eye and can give essential diagnostic information to the doctor.

Several drawbacks are however present in the known

25 apparatuses. A first problem is that the objects to be

observed within the image field (single cells or aggregates, etc.) often do not present the same brightness throughout the whole area of the image. This is mainly due to the position they occupy with respect

5   to the image's centre, which has an higher brightness, or to the eye's section under examination, which may not wholly intecepts the object.

A further drawback concerns the way the acquired image is processed by the computer. It may be necessary,

10 in some cases, to quantitatively evaluate physical and geometrical characteristics of the observed object, in order to achieve better diagnostic information. A typical example is the case of pharmacological trials regarding the corneal keratocytes and other components

15 of the corneal stroma. In such a case, the known devices do not allow a correct quantification of the requested geometrical parameters to be made, particularly for highly irregularly shaped objects such as the ones named above, with the consequence that the outcome of the

20 analysis may be incorrect or even misleading. There is therefore a need of improved methods and apparatuses that allow a correct quantification of the morphometric parameters of any item for which such quantification is requested.

25   The present invention addresses the above and other

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problems and solve them with a method and an apparatus
as depicted in the attached claims.

Further characteristics and the advantages of the
method and confocal microscopy apparatus for analyizing

5   living eye' images according to the present invention
will become clear from the following description of a
preferred embodiment thereof, given by way of non-
limiting example, with reference to the appended
drawings, in which:

10   Figure 1 is a schematic view of the apparatus
according to the invention;

Figure 2 is a schematic view of the optical
assembly of the apparatus of figure 1;

Figure 3 is a flow chart illustrating the method of

15   the invention.

The method of the invention allows one to analyse
and metrically quantify an object's image, particularly
the image of an object having irregular contour, whose
Euclidean dimensions are not representative of the

20   actual dimensions of the object. Even if the specific
example shown herein below is concerned with the direct
living eye's observation through a LSO technique, this
kind of objects recur often when analyising a biological
specimen.

25   With the term "biological specimens" it is herein

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intended any kind of biological sample taken from the human, animal or plant body (such as a tissue or cell sample) that can be analysed by means of Laser Scanning Confocal Microscopy or Laser Scanning Ophtalmoscopy

5   apparatuses.

The example that will be described hereinafter concerns a system 1 for acquiring and processing an image comprising a confocal scanning microscope 2. The microscope 2 is preferably of the type that allow

10   magnification from 50x up to 1000x.

The microscope 2 is provided with an object glass 8, at least one eyepiece 4 and at least one photo-video port 5 for camera attachment. To this latter, electronic image acquisition means 6, in particular a photo/video

15   camera, are operatively connected. Preferably, such electronic image acquisition means 6 are a digital camera, having more preferably a resolution of at least 1.3 Megapixels.

The confocal scanning microscope 2 is equipped with

20   a light source 3 which can be a halogen lamp or a laser beam source. Between the light source 3 and the photo-video port 5, along the light path, a slidable slit system 9 is located. A first slit 9' is positioned between the light source 3 and the object glass 8, so

25   that a slit-shaped light beam is projected onto the

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patient's cornea. Suitably, a first converging lens 10a
is interposed between the light source and the first
slit 9', while a mirror system 11a directs the slit-
shaped light beam to pass through a first half of the
5   object glass 8.

The light reflected by the patient's cornea pass
through the second half of the object glass 8 and then
through a second slit 9'' to the photo-video port 5.
Again, a mirror system 11b is suitably located in order
10   to direct the reflected light collected by the object
glass 8 to the second slit 9'' and a second converging
lens 10b converges the collected light to the said
photo-video port 5.

The slits 9', 9'' are slidable in the x, y plane so
15   that scanning of a cornea surface or section is
effected. The object glass 8 is able to move along the z
axis, in order to make a scanning along the depth of the
cornea. This allows a 3D-image of the patient's cornea
region to be acquired.

20   The electronic image acquisition means 6 are
operatively connected with a processing system 7. The
processing system 7 may be realized by means of a
personal computer (PC) comprising a bus which
interconnects a processing means, for example a central
25   processing unit (CPU), to storing means, including, for

example, a RAM working memory, a read-only memory (ROM) - which includes a basic program for starting the computer -, a magnetic hard disk, optionally a drive (DRV) for reading/writing opticasl disks (CD-RWs),

5   optionally a drive for reading/writing floppy disks. Moreover, the processing system 7 optionally comprises a MODEM or other network means for controlling communication with a telematics network, a keyboard controller, a mouse controller and a video controller. A

10   keyboard, a mouse and a monitor 12 are connected to the respective controllers. The electronic image acquisition means 6 are connected to the bus by means of an interface port (ITF). The slit system 9 and the object glass 8 are also connected to the bus by means of a

15   control interface port (CITF) by which the movement of both the slit system and the object glass along the Cartesian axis is governed. A joystick 13 may also be provided in order to manually control the positioning of the object glass 8.

20   A program (PRG), which is loaded into the working memory during the execution stage, and a respective data base are stored on the hard disk. Typically, the program (PRG) is distributed on one or more optical disks CD-ROMs for the installation on the hard disk.

25   Similar considerations apply if the processing

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system 7 has a different structure, for example, if it
is constituted by a central unit to which various
terminals are connected, or by a telematic computer
network (such as Internet, Intranet, VPN), if it has
5 other units (such as a printer), etc.. Alternatively,
the program is supplied on floppy disk, is pre-loaded
onto the hard disk, or is stored on any other substrate
which can be read by a computer, is sent to a user's
computer by means of the telematics network, is
10 broadcast by radio or, more generally, is supplied in
any form which can be loaded directly into the working
memory of the user's computer.

Coming now to the description of the analysis
procedure, the patient is positioned in front of the
15 microscope 2, so that the patient's eye is aligned with
the object glass 8. The object glass is spread with a
drop of a suitable ophthalmic gel and is then caused to
approach the patient's cornea up to a point that the eye
is wetted by the gel but the glass does not contact it.
20 At this point the scanning can be started until the
whole acquisition procedure is terminated.

Once the images acquisition has been completed, the
processing system 7 can perform the data elaboration
routines according to the preferred embodiment of the
25 invention, as will be depicted herein after.

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It is pointed out that some or all of the steps of the method of the invention can be performed by the computer system 7 by executing the program PRG.

The method of the invention provides for the
5   calculation of several parameters that can be of pivotal clinical significance.

In summary, the method of the invention is a method of processing digital images comprising one or more objects to be quantified, the said method comprising the
10   following main stages:

-       normalization of the digital images;

-       quantization of the images to one bit, further comprising at least one of the following stages:

-       calculation from the said images quantized to
15   one bit of the perimeter, area and/or fractal dimension of the said one or more objects to be quantified;

-       reconstruction from the said images quantized to one bit of a 3D-image of the said one or more objects to be quantified, and/or

20   -   calculation from the said normalized images of the fractal dimension of the overall image.

The stages which are part of the method of the invention will be now described in more details.

The first stage of the method of the invention is
25   the stage of <u>image normalization</u>. Image normalization is

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a known procedure which is often applied to digital images. However, as said above, when the observed eye's section contains several objects to be analysed (cells and the like), these objects do not always present the

5   same brightness throughout the image, the image's centre having an higher brightness than the contour. It has been found that the known normalization procedures utilizing parabolic functions do not serve the scope of the present invention, due to the described lack of

10   uniformity of the brightness in the different image's areas. The inventors of the present application have therefore provided a new routine which is called progressive image normalization (NORM stage).

Before starting the image normalization routine it

15   may be necessary to apply to the image a digital linear filter in order to remove the background noise. These filters are of the type conventionally used in image processing and can be used to remove isolated points. In the worse cases, a Gaussian filter can be used.

20   Once the image has been cleaned, if necessary, from the noise, the progressive image normalization can be started.

This stage is an iterative procedure which comprises the following steps:

25   1a) dividing the image into quadrants (typically,

four quadrants);

2a) calculating the mean value of intensity of the pixels belonging to each quadrant;

3a) calculating the mean value of intensity for the quadrants as a mean of the calculated means of step 2a);

4a) setting for each quadrant the mean value of intensity calculated according to step 3a) by performing one of adding or subtracting a same intensity value to each pixel inside a quadrant in order to maintain the original $\Delta_{intensity}$ among the pixels inside a same quadrant;

5a) determining for each quadrant the max and the min values of intensity of the pixels and calculating for each pixel an extended intensity value (EI) which derives from the stretching of the digital values inside the range of the possible digital values. The range of the possible digital values is 0-256. Maximum stretching is obtained by an extension of the intensity values in the whole 0-256 range. However, intermediate extensions are possible. Preferably, the said EI value is calculated by means of the following algorithm:

$$EI_{pixel} = (I_{pixel} - I_{min}) \times N / (I_{max} - I_{min})$$

wherein I_{pixel} is the intensity of each pixel of a given quadrant, I_{min} is the min value of intensity of the

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pixel inside the said quadrant, I_{max} is the max value of intensity of the pixel inside the same quadrant and N is an integer more than 1 and up to 255, preferably 255;

6a) setting for each pixel the EI_{pixel} calculated
5   according to step 5a);

7a) reiterating steps 1a) to 6a) up to a preset quadrant side length.

The preset quadrant side length depends on the dimension of the objects to be detected and preferably
10   will be approximately half length of the minor side of the object.

Step 5a) is also called as an extension of the pixels' intensity to a 0-255 scale and is helpful in order to improve the contrast inside the image. In some
15   instances, steps 5a) and 6a) can be skipped.

According to a preferred embodiment of the invention, the normalization stage is performed according to the following procedure:

1b) dividing the image into quadrants (typically,
20   four quadrants);

2b) determining for each quadrant the max and the min values of intensity of the pixels and calculating for each pixel an extended intensity value (EI) which derives from the stretching of the digital values inside
25   the range of the possible digital values. As said

before, the EI value can be calculated by means of the following algorithm:

$$EI = (I_{pixel} - I_{min}) \times N / (I_{max} - I_{min})$$

wherein I_{pixel} is the intensity of each pixel of a

5   given quadrant, I_{min} is the min value of intensity of the pixel inside the said quadrant, I_{max} is the max value of intensity of the pixel inside the same quadrant and N is an integer more than 1 and up to 255, preferably 255;

3b)   storing the EI_{pixel} value for each pixel of each

10   quadrant in a data structure;

4b)   reiterating steps 1b) to 3b) up to a preset quadrant side length in order to obtain for each pixel a set of intensity values in the data structure;

5b)   calculating for each pixel the mean of the

15   intensity values of the set stored in the data structure and setting the calculated mean value to the respective pixel.

Again, the preset quadrant side length depends on the dimension of the objects to be detected and

20   preferably will be approximately half length of the minor side of the object.

The routine depicted in steps 1b) to 5b) allows the processing system 7 to perform the whole calculation faster.

25   The second stage of the method of the invention is

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the stage of image elaboration (IMA-EL stage). This stage is performed by quantizing the image to "1 bit" in order to select image's regions on which further calculations are performed. The IMA-EL stage is
5 accomplished according to the following steps:

1c) considering a parameter for each pixel;

2c) comparing said pixel's parameter with a preset threshold value or threshold range for said parameter;

3c) selecting a cluster of active pixels and a
10 cluster of inactive pixels on the base of said comparison.

Said pixel's parameter is preferably brightness intensity (black and white images) or digital colour value. Said preset threshold value or range for said
15 parameter will mainly depend upon the kind of object that should be detected. Selection of such threshold values or ranges can be made in any case by the skilled man, for the particular case, without excercize of any inventive skill. For example, if the object whose image
20 has to be acquired is the corneal stroma (B & W image) the range of intensity values is 128-255.

Once the digital image has been quantized to 1 bit, the method of the invention provides for a stage of metrical processing of the image which is made on its
25 turn of different stages that will be depicted herein

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below.

The next stage of the invention method is thus the stage of object's metrical quantification (QUANT stage).

This stage has been set up for improving metrical quantification of the morphometric parameters of irregularly shaped objects, that can not be metered by the usual Euclidean geometry. The microscopic observation of either a normal or abnormal, such as pathological, component of a given organ, particularly an eye, is amazing because of the new irregularities that appear at any magnification (scale of observation). As the extension form of the image of the samples changes, the new irregular details are given measures and dimensions that are independent at each magnification and can not be arranged in a single linear system. Because of this characteristic, which is due to the scabrousness of the external surface of the object to be observed, the visible details, as well as those that can not be visually identified, make all objects with an irregular surface hardly measurable by means of traditional computer-aided morphometry.

The classical morphometry tackles the problem of measuring natural objects by approximating their irregular outlines and rough surfaces to rectilinear outlines and plane surfaces.

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Irregular objects were defined "fractal" by Benoit Mandelbrot since, in spite of the fact that their shape changes as a function of magnification, they retain the features of their irregularity at all spatial scales. Although the pieces (not fractions) into which they can be divided are not equal, they preserve the similitude of their irregularity. This property of the parts into which irregular objects can be divided is called "self-similarity". Since the shape of such objects depends on the magnification at which their image is observed, any quantitative metering of the dimensions of the object is a function of the magnification scale. The fractal dimension indicates therefore the "self-similarity" of the fractal pieces of an irregular body and, at each scale, defines the characteristics of the reference means used to measure the physical and geometrical parameters of the observed irregular object.

The first step of the QUANT stage is the calculation of the area of the object under examination. The unit of measurement may be μm^2 or pixel.

The area A of the object under examination is thus calculated by counting the number of pixels belonging to the cluster of active pixels selected according to the previous IMA-EL stage.

The second step of the QUANT stage is the

calculation of the perimeter P of the object under
investigation. This step is performed by i) selecting
the object contour's pixels, and ii) applying to such
selected pixels the perimeter calculation's algorithm

5   according to S. Prashker method (Steve Prashker, An
Improved Algorithm for Calculating the Perimeter and
Area of Raster Polygons, GeoComputation, 1999, which is
herein incorporated by reference). According to the
Prashker's method, each active pixel's surroundings are

10 taken into consideration, i.e. the eight pixels around
the pixel under examination. To each active pixel is
given a "perimeter value", whose sum is the overall
perimeter P of the object. If, for example, an internal
pixel is considered (i.e. a pixel totally surrounded by

15 active pixels, thus not belonging to the perimeter of
the object), to such a pixel is given a "perimeter
value" of 0. If a perimeter's pixel is connected with
two other pixels through the corners along a diagonal
line, the "perimeter value" is $\sqrt{2}$ pixels. If the

20 considered active pixel is connected to one pixel
through the corner and to another pixel by a side, the
"perimeter value" will be (0.5 + $\sqrt{2}/2$) pixels. If an
active pixel is connected to the two adjacent pixels
through its sides, the "perimeter value" will be then 1

25 pixel and so on.

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Given the considerable irregularity of the perimeter of the object under examination, an evaluation of its fractal dimension D_P is made. Similarly, the estimate of the fractal dimension of the area of the

5   selected structure is indicated by the symbol D_A. Both of these fractal dimensions can be automatically determined using the known "box-counting" algorithm.

According to the "box-counting" method, the fractal dimension D is given by the mathematical formula

10    $D = \lim(\varepsilon \to 0) \ [\log N(\varepsilon)/\log(1/\varepsilon)]$

wherein ε is the length of the side of the boxes of the grid in which the object's image has been divided and $N(\varepsilon)$ is the number of boxes necessary to completely cover the outline (D_P) or the area (D_A), respectively,

15   of the measured object. The length ε is expressed in pixel or μm and, in the present calculation method, ε tends to 1 pixel.

The next stage of the invention method is thus the stage of dimensional calculation (DIM-CLC stage).

20   In order to avoid difficulties in such a calculation, the fractal dimensions D_P and D_A are approximated as the slope of the straight line obtained by putting in a Cartesian axis system the parameters $\log N(\varepsilon)$ versus $\log(1/\varepsilon)$.

25   In practice, the method used to determine D_P

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comprises the following steps, performed by the CPU of the processing system 7:

a)   dividing the image of the object into a plurality of grids of boxes having a side length ε, in

5   which ε varies from a first value substantially corresponding to the side of the box in which said object is inscribed and a predefined value which is a fraction of said first value,

b)   calculating a value of a logarithmic

10 function of $N(\varepsilon)$, in which $N(\varepsilon)$ is the number of boxes necessary to completely cover the perimeter (P) of the object and of a logarithmic function of $1/\varepsilon$ for each ε value of step a), thus obtaining a first set of values for said logarithmic function of $N(\varepsilon)$ and a second set

15 of values for said logarithmic function of $1/\varepsilon$,

c)   calculating the fractal dimension D_P as the slope of the straight line interpolating said first set of values versus said second set of values of step b).

20 The same method is applied for calculating the fractal dimension D_A, with the only difference that, in this case, $N(\varepsilon)$ is the number of boxes of side ε that completely cover the area of the object to be quantified.

25 The fractal dimensions D_P and D_A of the single

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objects are a numerical index of the irregularity of the object itself, i.e. whether the object is more or less irregularly shaped. This can give a useful indication to the clinician about the pathological condition of the

5   patient.

Since an ocular image of the stroma evidences a multiplicity of small objects (cells) which give an indication of the pathological degree of the patient, it is important for a metrical analysis of the stroma to

10   identify all the objects observed through the ophtalmoscope. A further stage of the method of the invention is therefore the <u>stage of object's sorting</u> (SORT stage) which includes the following steps:

1d) scanning of the image quantized to "1 bit"

15   along a predefined direction on a x, y axis system;

2d) selecting a first active pixel along said direction of scanning, said active pixel being identified by a first set of x, y values, said first active pixel belonging to a first object's image;

20   3d) performing on said first selected active pixel a search routine in the positions next to said selected pixel on the direction's line;

4d) iterating step 3d) until an inactive pixel is found;

25   5d) assigning to each active pixel selected

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according to such steps 3d) and 4d) a set of x, y values, saving them in the storing means of the processing system 7 (all of such pixels will have the same y value and x values in progressive order) and

5   switching said pixels from active to inactive in the object's image;

6d)   evaluating for each pixel selected according to steps 3d), 4d) and 5d) the two next pixels in the direction ortogonal to the said scanning direction and

10   selecting the active pixels;

7d)   performing, for each of said active pixels selected according to step 6d), the routine of steps 3d) to 5d);

8d)   iterating steps 6d) and 7d) until all of the

15   connected pixels belonging to the same object have been saved;

9d)   repeating steps 1d) and 2d) until a first active pixel of a further object's image is found;

10d) repeating steps 3d) to 9d) until the whole

20   image has been scanned.

Said predefined direction in step 1d) is preferably from left to right starting from top to bottom.

The procedure depicted in steps 1d) to 10d) above allows to identify objects made up from 4-connected

25   pixels, i.e. wherein the pixels have one side in common.

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For sorting also 8-connected pixel objects, step 6d) of the above procedure is modified as follows:

6d) evaluating for each pixel selected according to steps 3d), 4d) and 5d) the two next pixels in the direction ortogonal to the said scanning direction and the two pixels adjacent to each of these latter pixels on the parallel line adjacent to the direction's line and selecting the active pixels.

The procedure is then prosecuted according to steps 7d) to 10d).

The procedure herein above depicted is a semi-recursive method which allows, with respect to the standard recursive methods of the art, shorter execution time and less memory request. In fact, taking into consideration an image made up of NxM active pixels, only M recursive calls are necessary, while according to the prior art methods the number of recursive calls would be NxM −1.

After the SORT stage, the method of the invention may perform the following steps:

1e) calculating the area of each object identified according to the SORT stage by counting the number of pixels belonging to said object's image and multiplying it for the area of each pixel;

2e) counting the number of objects and calculating

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its density;

3e) calculating the mean area of the objects by adding the areas calculated according to step 1e) of all the objects sorted and dividing the total area by the number of objects obtained according to step 2e).

The method of the invention also allows the calculation of a parameter known as "rugosity" which gives an indication of the unevenness of the surface of the object to be quantified (typically, a cell structure). The parameter w indicating the degree of "rugosity" of the selected object can be calculated by means of the following algorithm:

$$w = \frac{Pf}{2\sqrt{Af \cdot \pi}} - R \qquad (III)$$

wherein Pf is the perimeter, Af is the area of the object and R is the "roundness coefficient" of the object. R is on its turn calculated with the following algorithm

$$R = \frac{Pe}{2\sqrt{Ae.\pi}} \qquad (IV)$$

wherein Pe is the perimeter of the ellipse in which the measured object is inscribed and Ae its area.

A further stage of the method of the invention is the <u>stage of surface quantification</u> (S-QUANT stage).

This stage provides for a metrical evaluation of the "surface" of the whole image. This helps achieving a better picture of the distribution and shape of the

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various single objects (cells and the like) inside the cornea and thus improving the diagnostic outcome.

The base concept is that the image can be seen as a tridimensional surface. The grey scale values of the
5   pixels in the image are an index of how much the observed object extends along the axis orthogonal to the image (z axis). In other words, the digital image appears as a "hill cluster" whose surface dimension can be calculated as a fractsl dimension. For these reasons,
10 the S-QUANT stage is performed on the image normalized according to the routine described above, but before the said IMA-EL stage.

In this case too the fractal dimension of the surface can be calculated by using the "box counting"
15 methodology, which is however adapted for set of values x, y, z, i.e. in the three dimensions.

The S-QUANT stage comprises the following steps:

1f) dividing the image in a x, y bidimensional mesh with n x n boxes of side l;

20 2f) dividing the 0-256 grey scale into n subregions having each a 256/n value;

3f) calculating for each box of the x, y bidimensioanl mesh the min and max value of the pixels contained therein and of the pixels that contour the
25 box;

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4f) calculating how many subregions of 256/n value are included between the min and max values of the pixels of each box;

5f) calculating the number N(l) of tridimensional boxes of side l that intercepts the image's surface as a sum of the subregions of all the boxes calculated according to step 4f);

6f) reiterating steps 1f) to 5f) with a side length l' less than l;

7f) by repeating step 6f), generating a first set of values of a logarithmic function of 1/l and a second set of values of a logarithmic function of N(l);

8f) calculating the fractal dimension of the image's surface as the slope of the straight line interpolating said first set of values versus said second set of values of step 7f).

The calculation of the fractal dimension of the surface provides a numerical index of the image's complexity, i.e. the distribution of the cells in the observed tissue, which can be correlated with the pathological condition of the patient.

As said before, the LSO technique provides for a 3D-reconstruction of the image which is made possible by the scanning in the z direction of the observed item. In the present specific example, a picture of several

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sections of the observed cornea is taken and the several acquired 2D-images are reconstructed to form a tridimensional image. The so reconstructed 3D-image is helpful in order to have an overall picture of the

5   observed tissue and thus to identify type, number and density of the cells that are contained therein.

Therefore, the method of the present invention also comprises the volume analysis.

The first stage of the volume analysis is the stage

10 of 3D-reconstruction (3D-R stage). This stage is performed on the image once it has been subjected to the IMA-EL stage.

According to the invention procedure, the 3D-image is obtained by overlapping the 2D-images collected for

15 each section of the examined tissue. However, due to even minor movements of the observed eye during the analysis performance, there can be some misalignement between one 2D-image and the subsequent 2D-image in the direction of scanning. The method of the invention thus

20 provides for an adjustement of the offset between the overlapped images.

The 3D-R stage comprises the following steps:

1g) overlapping each image with the subsequent image along the z axis;

25   2g) minimizing the difference of brightness and/or

colour intensity between overlapping pixels by shifting along the x axis and/or the y axis an image with respect to each other;

3g) repeating steps 1g) and 2g) for each pair of adjacent images.

After the 3D-image has been reconstructed, it is possible to proceed with the counting of the number of items (typically cells) that are contained in the observed tissue, as well as with the calculation of their density. These parameters too are of utmost importance to achieve meaningful diagnosis results.

Counting of the cells is performed by means of the object counting stage (O-COUNT stage), which comprises the following steps:

1h) scanning of the 3D-image quantized to "1 bit" along a predefined direction on a x, y axis system;

2h) selecting a first active pixel along said direction of scanning, said active pixel being identified by a first set of x, y values, said first active pixel belonging to a first object's image;

3h) performing on said first selected active pixel a search routine in the positions next to said selected pixel on the direction's line;

4h) iterating step 3h) until an inactive pixel is found;

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5h) assigning to each active pixel selected according to such steps 3h) and 4h) a set of x, y values, saving them in the storing means of the processing system 7 (all of such pixels will have the same y value and x values in progressive order) and switching said pixels from active to inactive in the object's image;

6h) evaluating for each pixel selected according to steps 3h), 4h) and 5h) the two next pixels in the coplanar direction orthogonal to the said scanning direction and the two next pixels along the z axis, in the directions +z and -z, and selecting the active pixels;

7h) performing, for each of said active pixels selected according to step 6h), the routine of steps 3h) to 5h);

8h) iterating steps 6h) and 7h) until all of the connected pixels belonging to the same object have been saved;

9h) repeating steps 1h) and 2h) until a first active pixel of a further object's image is found;

10h) repeating steps 3h) to 9h) until the whole image has been scanned;

11h) counting of the number of the objects sorted according to steps 1h) to 10h).

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Said predefined direction in step 1h) is preferably from left to right starting from top to bottom.

The search of the active pixels in the directions +z and -z is performed by overlapping the images in sequence.

The procedure depicted in steps 1h) to 10h) above allows to identify objects made up from 4-connected pixels, i.e. wherein the pixels have one side in common.

For sorting also 8-connected pixel objects, step 6h) of the above procedure is modified as follows:

6h) evaluating for each pixel selected according to steps 3h), 4h) and 5h) the two next pixels in the coplanar direction orthogonal to the said scanning direction and the two next pixels along the z axis, in the directions +z and -z, and the two pixels adjacent to each of these pixels on the parallel line adjacent to the direction's line and selecting the active pixels.

The procedure is then prosecuted according to steps 7h) to 10h).

The procedure herein above depicted is a semi-recursive method which allows, with respect to the standard recursive methods of the art, shorter execution time and less memory request.

Once the number of objects, namely cells, contained in the examined tissue has been determined according to

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the above procedure, the objects' density is easily determined as the total number of objects over the whole 3D-image volume:

$$d = N_{objects}/V_{image}$$

5   wherein the image's volume is calculated as the number of sections multiplied for the interval thickness among the sections, multiplied for the extension (area) of the section.

The next stage of the method of the invention is 10   the <u>stage of volume calculation</u> (V-CLC stage). According to this stage the volume of the objects contained in the examined tissue is determined.

The V-CLC stage comprises the following steps:

1i) calculating the area of each object in a first 15   2D-image corresponding to a first object's section;

2i) multiplying the area calculated according to step 1i) for the distance between the said first section's image and the subsequent section's image, taken in the z direction of scanning, wherein an image 20   of the same object is contained;

3i) reiterating steps 1i) and 2i) for each section's image in the order.

The overall volume of the objects in the examined tissue is determined as the sum of the single volumes 25   calculated according to the above procedure.

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The area calculation according to step 1i) is preferably made by counting the number of active pixels belonging to the same object and then multiplying for the area of the pixel. The object is identified as

5 depicted in the O-COUNT stage, so that each object is given a set of x, y and z values.

The distance between each section's image and the subsequent one is a known parameter in the confocal microscopy technique.

10 The above volume was calculated by approximating the objects' volume to that of a substantially cylindrical solid. However, by approximating it to a frustum of cone, the volume being calculated as:

$$v = 1/3d(A + a + \sqrt{A.a})$$

15 wherein d is the known distance between the two sections, A is the area of the first object's section and a is the area of the second object's section.

The mean volume of the objects is finally given by dividing the overall volume for the number of objects as

20 calculated before.

From what has been said above, it is clear that the calculation method of the invention represents an improvement if compared with the known methods. The fractal geometry offers mathematical models derived from

25 the infinitesimal calculus that, when applied to

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Euclidean geometry, integrate the figures of the morphometrical measurements of natural and irregular objects, thus making them closer to the actual values. Dimensional calculation using the fractal geometry gives numerical indexes (fractal dimensions) for both the single objects (index of the space distribution of the object's area/volume) and the image as a whole (index of the space distribution of the objects in the observed tissue). This allows the clinician to compare numerical values of the patient with standardised values, thus arriving immediately and with repeatable accuracy to the diagnosis of the pathological condition of the patient. This is believed to be a dramatic improvement on the prior art diagnostic methods, wherein only a visual and qualitative analysis of the patient's eye image was available in order to make the diagnosis.

Naturally, only some specific embodiments of the method and apparatus for analyizing biological tissue specimens according to the present invention have been described and a person skilled in the art will be able to apply any modification necessary to adapt it to particular applications without, however, departing from the scope of protection of the present invention.

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CLAIMS

1.   Method of processing digital images comprising one or more objects to be quantified, the said method comprising the following main stages:

5   -   normalization of the digital images;

-      quantization of the images to one bit, further comprising at least one of the following stages:

-      calculating, from the said images quantized to one bit, the perimeter, area and/or fractal dimension of

10   the said one or more objects to be quantified;

-      reconstructing, from the said images quantized to one bit, a 3D-image of the said one or more objects to be quantified, and/or

-      calculating, from the said normalized images,

15   the fractal dimension of the overall image.

2.   Method according to claim 1, the said method comprising a stage of image's normalization (NORM) which comprises the following steps:

1)   dividing the image into quadrants;

20   2)   calculating the mean value of intensity of the pixels belonging to each quadrant;

3)   calculating the mean value of intensity for all the quadrants as a mean of the calculated means of step 2);

25   4)   setting for each quadrant the mean value of

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intensity calculated according to step 3) by performing one of adding or subtracting a same intensity value to each pixel inside a quadrant in order to maintain the original $\Delta_{intensity}$ among the pixels inside a same

5   quadrant;

5)   reiterating steps 1) to 4) up to a preset quadrant side length.

3.   Method according to claim 2, wherein the said preset quadrant side length is approximately half length

10   of the minor side of the said one or more objects to be quantified.

4.   Method according to any one of claims from 1 to 3, wherein the said digital image has been acquired by a confocal microscopy.

15   5.   Method according to claim 4, wherein the said confocal microscopy is a Laser Scanning Confocal Microscopy (LSCM) or a Scanning Ophtalmoscopy.

6.   Method according to any one of claims from 1 to 5, which comprises the following steps:

20   1a)   dividing the image into four quadrants;

2a)   calculating the mean value of intensity of the pixels belonging to each quadrant;

3a)   calculating the mean value of intensity for the four quadrants as a mean of the four calculated

25   means of step 2a);

4a) setting for each quadrant the mean value of intensity calculated according to step 3a) by performing one of adding or subtracting a same intensity value to each pixel inside a quadrant in order to maintain the

5 original $\Delta_{intensity}$ among the pixels inside a same quadrant;

5a) determining for each quadrant the max and the min values of intensity of the pixels and calculating for each pixel an extended intensity value (EI) which

10 derives from the stretching of the digital values inside the range of the possible digital values;

6a) setting for each pixel the EI_{pixel} calculated according to step 5a);

7a) reiterating steps 1a) to 6a) up to a preset

15 quadrant side length.

7. Method according to claim 6, wherein the said step 5a) of calculating the EI value of the pixels is performed by means of the following algorithm:

$$EI_{pixel} = (I_{pixel} - I_{min}) \times N / (I_{max} - I_{min})$$

20 wherein I_{pixel} is the intensity of each pixel of a given quadrant, I_{min} is the min value of intensity of the pixel inside the said quadrant, I_{max} is the max value of intensity of the pixel inside the same quadrant and N is an integer more than 1 and up to 255, preferably 255.

25 8. Method according to any one of claims from 1

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to 5, wherein the said normalization stage comprises:

1b)   dividing the image into quadrants;

2b)   determining for each quadrant the max and the min values of intensity of the pixels and calculating

5   for each pixel an extended intensity value (EI) which derives from the stretching of the digital values inside the range of the possible digital values;

3b)   storing the EI_{pixel} value for each pixel of each quadrant in a data structure;

10   4b)   reiterating steps 1b) to 3b) up to a preset quadrant side length in order to obtain for each pixel a set of intensity values in the data structure;

5b)   calculating for each pixel the mean of the intensity values of the set stored in the data structure

15   and setting the calculated mean value to the respective pixel.

9.   Method according to claim 8, wherein the said step 5a) of calculating the EI value of the pixels is performed by means of the following algorithm:

20   $EI_{pixel} = (I_{pixel} - I_{min}) \times N / (I_{max} - I_{min})$

wherein I_{pixel} is the intensity of each pixel of a given quadrant, I_{min} is the min value of intensity of the pixel inside the said quadrant, I_{max} is the max value of intensity of the pixel inside the same quadrant and N is

25   an integer more than 1 and up to 255, preferably 255.

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10. Method according to any one of claims from 1 to 9, further comprising a stage of image elaboration (IMA-EL stage) to quantize the image to "1 bit".

11. Method according claim 10, wherein the said
5 IMA-EL stage comprises the following steps:

1c) considering a parameter for each pixel;

2c) comparing said pixel's parameter with a preset threshold value or threshold range for said parameter;

3c) selecting a cluster of active pixels and a
10 cluster of inactive pixels on the base of said comparison,

wherein said pixel's parameter is preferably brightness intensity (black and white images) or digital colour value.

15 12. Method according to any one of claims from 1 to 11, further comprising a stage of image quantification which comprising at least one of the following steps:

- calculating the area A of the object under
20 examination by counting the number of pixels belonging to the cluster of active pixels selected according to the previous IMA-EL stage;

- calculating the perimeter P of the object under examination by i) selecting the object contour's pixels,
25 and ii) applying to such selected pixels a perimeter

calculation's algorithm, wherein to each active pixel belonging to the object is given a "perimeter value", which is a function of the position of the active pixels adjacent to the pixel under examination, the sum of said

5   "perimeter values" being the overall perimeter P of the object.

13.   Method according to any one of claims from 1 to 12, further comprising a stage of object's sorting (SORT) for identifying objects made up from 4-connected

10   pixels, which includes the following steps:

1d)   scanning of the image quantized to "1 bit" along a predefined direction on a x, y axis system;

2d)   selecting a first active pixel along said direction of scanning, said active pixel being

15   identified by a first set of x, y values, said first active pixel belonging to a first object's image;

3d)   performing on said first selected active pixel a search routine in the positions next to said selected pixel on the direction's line;

20   4d)   iterating step 3d) until an inactive pixel is found;

5d)   assigning to each active pixel selected according to such steps 3d) and 4d) a set of x, y values, saving them in the storing means of the

25   processing system (7) and switching said pixels from

active to inactive in the object's image;

6d)   evaluating for each pixel selected according to steps 3d), 4d) and 5d) the two next pixels in the direction orthogonal to the said scanning direction and selecting the active pixels;

7d)   performing, for each of said active pixels selected according to step 6d), the routine of steps 3d) to 5d);

8d)   iterating steps 6d) and 7d) until all of the connected pixels belonging to the same object have been saved;

9d)   repeating steps 1d) and 2d) until a first active pixel of a further object's image is found;

10d)   repeating steps 3d) to 9d) until the whole image has been scanned.

14.   Method according to claim 13, wherein said predefined direction in step 1d) is from left to right starting from top to bottom.

15.   Method according to claim 13 or claim 14, wherein the stage of object's sorting according to steps 1d) to 10d) is performed for also identifying objects made up from 8-connected pixels, in said stage the step 6d) being modified as follows:

6d)   evaluating for each pixel selected according to steps 3d), 4d) and 5d) the two next pixels in the

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direction orthogonal to the said scanning direction and the two pixels adjacent to each of these latter pixels on the parallel line adjacent to the direction's line and selecting the active pixels.

5　　　16. Method according to any one of claims from 13 to 15, further comprising at least one of the following steps:

1e) calculating the area of each object identified according to the SORT stage by counting the number of

10　pixels belonging to said object's image and multiplying it for the area of each pixel; and/or

2e) counting the number of objects and calculating its density; and/or

3e) calculating the mean area of the objects by

15　adding the areas calculated according to step 1e) of all the objects sorted and dividing the total area by the number of objects obtained according to step 2e).

17. Method according to any one of claims from 1 to 16, further comprising a step of calculating a

20　parameter (w) indicating the degree of "rugosity" of the selected object, the said (w) parameter being preferably calculated by means of the following algorithm:

$$w = \frac{Pf}{2\sqrt{Af \cdot \pi}} - R$$

wherein Pf is the perimeter, Af is the area of the

25　object and R is the "roundness coefficient" of the

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object; wherein R is on its turn calculated with the following algorithm

$$R = \frac{Pe}{2\sqrt{Ae.\pi}}$$

wherein Pe is the perimeter of the ellipse in which
5   the measured object is inscribed and Ae its area.

18. Method according to any one of claims from 1 to 17, further comprising a stage of dimensional calculation (DIM-CLC) for calculating the fractal dimensions of perimeter and area of the observed
10 objects, wherein said fractal dimension of the perimeter (D_P) and said fractal dimension of the area (D_A) are determined according to the following steps:

a)   dividing the image of the object into a plurality of grids of boxes having a side length ε, in
15 which ε varies from a first value substantially corresponding to the side of the box in which said object is inscribed and a predefined value which is a fraction of said first value,

b)   calculating a value of a logarithmic
20 function of $N(\varepsilon)$, in which $N(\varepsilon)$ is the number of boxes necessary to completely cover the perimeter (P) or the area (A), respectively, of the object and of a logarithmic function of $1/\varepsilon$ for each ε value of step a), thus obtaining a first set of values for said
25 logarithmic function of $N(\varepsilon)$ and a second set of values

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for said logarithmic function of $1/\varepsilon$,

c)   calculating the fractal dimensions (D_P) or (D_A) as the slope of the straight line interpolating said first set of values for said logarithmic function

5   of $N(\varepsilon)$ for the perimeter (P) or the area (A), respectively, versus said second set of values of step b).

19.   Method according to any one of claims from 1 to 18, further comprising a stage of surface

10 quantification (S-QUANT) performed on the image normalized according to the NORM stage, the said stage comprising the following steps:

1f)   dividing the image in a x, y bidimensional mesh with n x n boxes of side l;

15   2f)   dividing the 0-256 grey scale into n subregions having each a 256/n value;

3f)   calculating for each box of the x, y bidimensioanl mesh the min and max value of the pixels contained therein and of the pixels that contour the

20 box;

4f)   calculating how many subregions of 256/n value are included between the min and max values of the pixels of each box;

5f)   calculating the number $N(l)$ of tridimensional

25 boxes of side l that intercepts the image's surface as a

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sum of the subregions of all the boxes calculated according to step 4f);

6f) reiterating steps 1f) to 5f) with a side length l′ less than l;

5   7f) by repeating step 6f), generating a first set of values of a logarithmic function of 1/l and a second set of values of a logarithmic function of N(l);

8f) calculating the fractal dimension of the image's surface as the slope of the straight line
10   interpolating said first set of values versus said second set of values of step 7f).

20. Method according to any one of claims from 1 to 19, further comprising a stage of 3D-reconstruction (3D-R) performed on the image subjected to the IMA-EL
15   stage, the said 3D-R stage comprising the following steps:

1g) overlapping each image with the subsequent image along the z axis;

2g) minimizing the difference of brightness and/or
20   colour intensity between overlapping pixels by shifting along the x axis and/or the y axis an image with respect to each other;

3g) repeating steps 1g) and 2g) for each pair of adjacent images.

25   21. Method according to claim 20, further

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comprising a stage of object counting (O-COUNT), which
comprises the following steps:

1h) scanning of the 3D-image quantized to "1 bit"
along a predefined direction on a x, y axis system;

5   2h) selecting a first active pixel along said
direction of scanning, said active pixel being
identified by a first set of x, y values, said first
active pixel belonging to a first object's image;

3h) performing on said first selected active pixel

10   a search routine in the positions next to said selected
pixel on the direction's line;

4h) iterating step 3h) until an inactive pixel is
found;

5h) assigning to each active pixel selected

15   according to such steps 3h) and 4h) a set of x, y
values, saving them in the storing means of the
processing system 7 (all of such pixels will have the
same y value and x values in progressive order) and
switching said pixels from active to inactive in the

20   object's image;

6h) evaluating for each pixel selected according to
steps 3h), 4h) and 5h) the two next pixels in the
coplanar direction orthogonal to the said scanning
direction and the two next pixels along the z axis, in

25   the directions +z and -z, and selecting the active

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pixels;

    7h) performing, for each of said active pixels selected according to step 6h), the routine of steps 3h) to 5h);

5   8h) iterating steps 6h) and 7h) until all of the connected pixels belonging to the same object have been saved;

    9h) repeating steps 1h) and 2h) until a first active pixel of a further object's image is found;

10   10h) repeating steps 3h) to 9h) until the whole image has been scanned;

    11h) counting of the number of the objects sorted according to steps 1h) to 10h).

    22. Method according to claim 21, wherein the said

15 predefined direction in step 1h) is from left to right starting from top to bottom.

    23. Method according to claim 21 or claim 22, for sorting also 8-connected pixel objects, wherein step 6h) of the procedure depicted in claim 21 is modified as

20 follows:

    6h) evaluating for each pixel selected according to steps 3h), 4h) and 5h) the two next pixels in the coplanar direction orthogonal to the said scanning direction and the two next pixels along the z axis, in

25 the directions +z and -z, and the two pixels adjacent to

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each of these pixels on the parallel line adjacent to the direction's line and selecting the active pixels.

24. Method according to any one of claims from 1 to 23, further comprising a stage of volume calculation (V-CLC) which comprises the following steps:

1i) calculating the area of each object in a first 2D-image corresponding to a first object's section;

2i) multiplying the area calculated according to step 1i) for the distance between the said first section's image and the subsequent section's image, taken in the z direction of scanning, wherein an image of the same object is contained;

3i) reiterating steps 1i) and 2i) for each section's image in the order.

25. Method according to claim 24, wherein the overall volume of the objects in the examined tissue is determined as the sum of the single volumes.

26. Method according to claim 24 or claim 25, wherein the volume is calculated as:

$$v = 1/3d(A + a + \sqrt{A.a})$$

wherein d is the distance between the two sections, A is the area of the first object's section and a is the area of the second object's section.

27. A system (1) for acquiring and processing an image including a confocal scanning microscope (2),

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electronic image acquisition means (6) operatively connected to said microscope (2), a processing system (7) operatively connected with said confocal scaning microscope (2) and said image acquisition means (6),

5   said processing system (7) comprising a processing unit (CPU), storing means which include a RAM working memory and a hard disk, said processing system (7) running a program (PRG) to perform a method according to any one of claims from 1 to 26.

10   28. A software program (PRG) to perform the method according to any one of claims from 1 to 26.

29. A computer readable support comprising a program (PRG) to perform the method according to any one of claims from 1 to 26.

15   30. Use of a system (1) according to claim 27 or claim 28, for performing a method as depicted in any one of claims from 1 to 26.
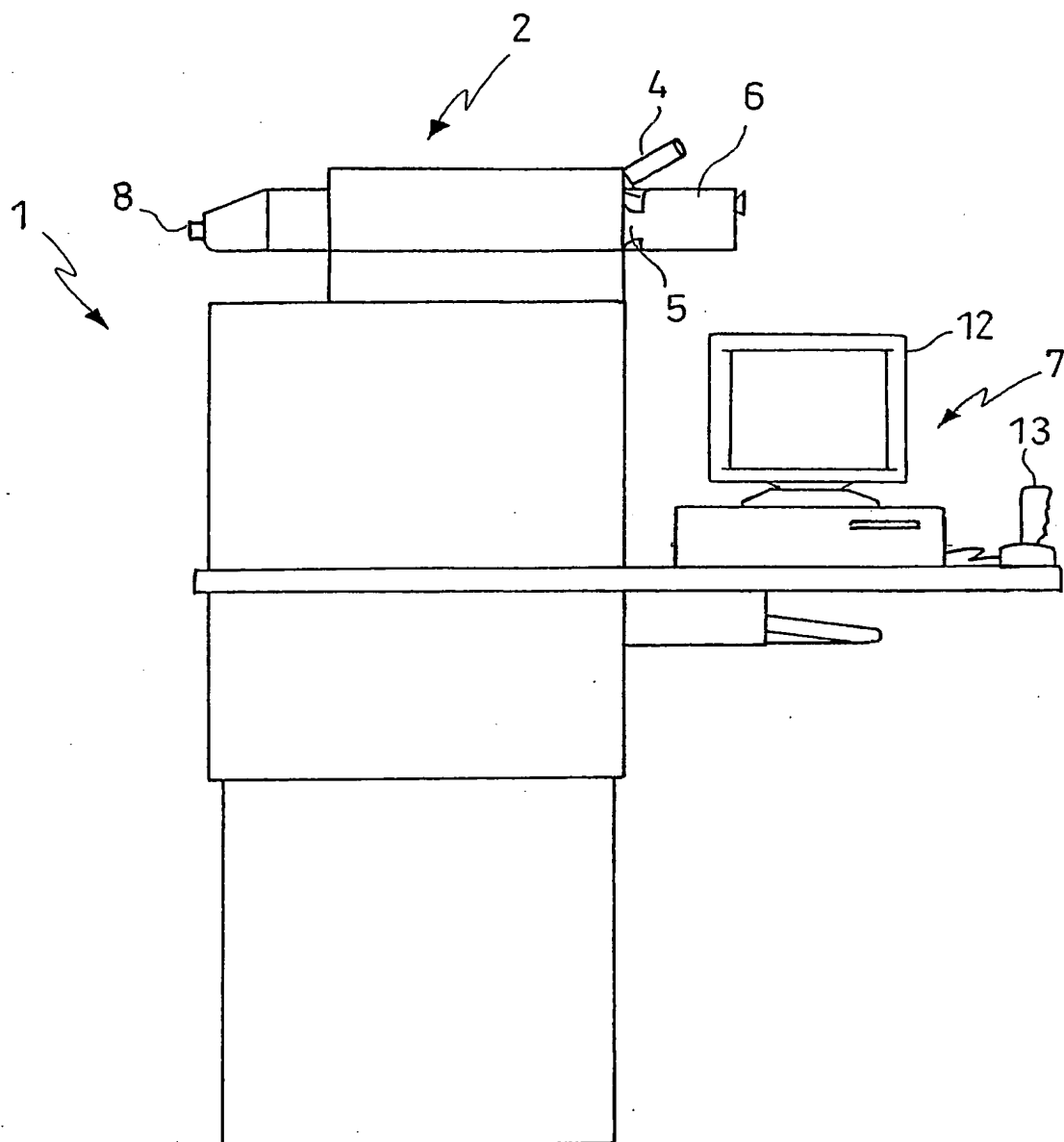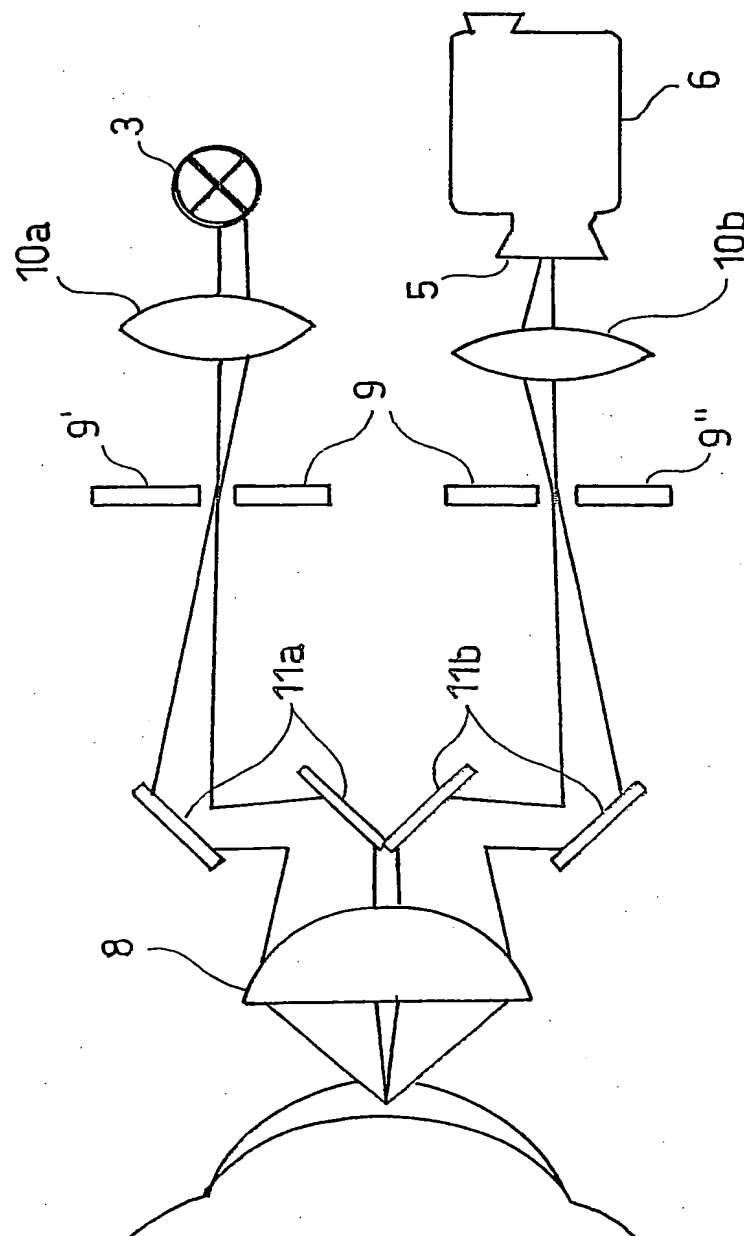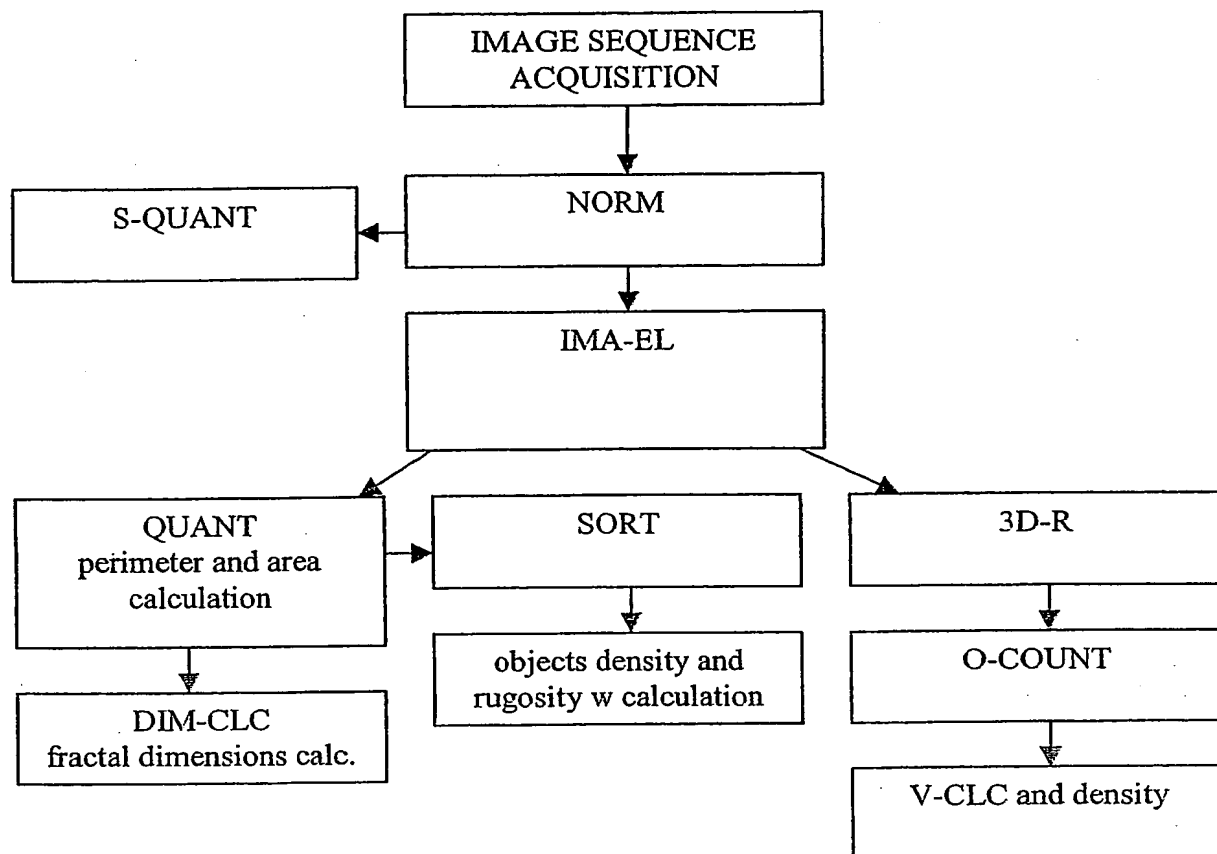
FIG.1

FIG.2

FIG. 3

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   G06T7/60   G06T7/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   G06T

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, INSPEC, IBM-TDB

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MERCHANT F A ET AL: "Semi-automatic morphological measurements of 2-D and 3-D microvascular images" PROCEEDINGS OF THE INTERNATIONAL CONFERENCE ON IMAGE PROCESSING (ICIP) AUSTIN, NOV. 13 - 16, 1994, LOS ALAMITOS, IEEE COMP. SOC. PRESS, US, vol. VOL. 3 CONF. 1, 13 November 1994 (1994-11-13), pages 416-420, XP010146065 ISBN: 0-8186-6952-7	1,4,5, 10,12, 27-30
A	the whole document	2,3,6-9, 11,13-26

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 March 2004	16/04/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Herter, J

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INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	the whole document	2,3,6-9, 11-26
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 5162990	A	10-11-1992	NONE	

(51) International Patent Classification⁷: G06T 7/60, 7/00

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(25) Filing Language: English

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(71) Applicants *(for all designated States except US)*: HU-MANITAS MIRASOLE S.p.A. [IT/IT]; Via Manzoni, 56, I-20089 Rozzano, Milano (IT). FONDAZIONE "MICHELE RODRIGUEZ" - ISTITUTO SCIENTIFICO PER LE MISURE QUANTITATIVE IN MEDICINA [IT/IT]; Via Ludovico di Breme, 79, I-20156 Milano (IT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: DIOGUARDI, Nicola [IT/IT]; Istituto Clinico Humanitas, Via Manzoni, 56, I-20089 Rozzano, Milano (IT). GRIZZI, Fabio [IT/IT]; c/o Istituto Clinico Humanitas, Via Manzoni, 56, I-20089 Rozzano, Milan (IT). RUSSO, Carlo [IT/IT]; c/o Istituto Clinico Humanitas, Via Manzoni, 56, I-20089 Rozzano, Milan (IT). FRANCESCHINI, Barbara [IT/IT]; c/o Istituto Clinico Humanitas, Via Manzoni 56, I-20089 Rozzano, Milano (IT). VINCIGUERRA, Paolo [IT/IT]; c/o Istituto Clinico Humanitas, Via Manzoni, 56, I-20089

Rozzano (IT). TORRES-MUNOZ, Ingrid [IT/IT]; c/o Istituto Clinico Humanitas, Via Manzoni, 56, I-20089 Rozzano (IT).

(74) Agents: LONG, Giorgio et al.; Jacobacci & Partners S.p.A., Via Senato, 8, I-20121 Milano (IT).

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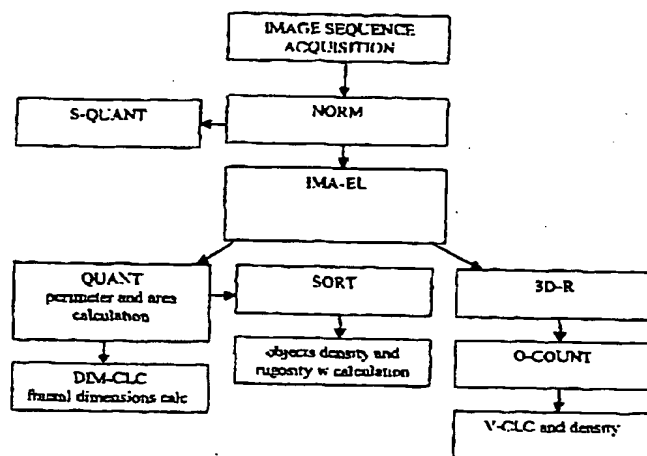
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(54) Title: METHOD AND APPARATUS FOR ANALYZING BIOLOGICAL TISSUES

(57) Abstract: The present invention relates to a method and an apparatus for processing images of irregularly shaped objects, such as biological specimens, in particular of human or animal origin, or images thereof. The metric quantification of a biological body part or tissue or of a material spot or aggregate of any origin, which is contained therein, is also performed by means of the invention method. In particular, the method of the present invention is applied to the "confocal microscopy" technique. In particular, the present invention relates to a method of processing digital images comprising one or more objects to be quantified, the said method comprising the following main stages:- normalization of the digital images; - quantization of the images to one bit, further comprising at least one of the following stages: - calculating, from the said images quantized to one bit, the perimeter, area and/or fractal dimension of the said one or more objects to be quantified; - reconstructing, from the said images quantized to one bit, a 3D-image of the said one or more objects to be quantified, and/or- calculating, from the said normalized images, the fractal dimension of the overall image.

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